F_0F_1-ATPASE AND DNA ENCODING THE SAME

BACKGROUND OF THE INVENTION

The present invention relates to a novel F_0F_1-ATPase, a DNA encoding the F_0F_1-ATPase, a method for producing the F_0F_1-ATPase, and a method for producing nucleoside 5'-triphosphate, using the F_0F_1-ATPase.

F_0F_1-ATPase plays principal roles in the biological energy metabolism, because the enzyme has an activity of generating adenosine 5'-triphosphate (ATP) as an energy source of organisms, by utilizing the gradient of proton concentration between the intramembrane and the extramembrane. Therefore, utilization of F_0F_1-ATPase enables us to develop a living thing with improved energy metabolism.

Herein, F_0F_1-ATPase is synonymous with H^+-ATPase.

Because it has been known that the activity of F_0F_1-ATPase varies, depending on the change of outer environment, such as pH, the utilization of F_0F_1-ATPase can provide a living thing adaptable to the change of outer environment [Mol. Microbiol., 33, 1152 (1999), J. Bacteriol., 176, 5167 (1994)].

F_0F_1-ATPase is a protein complex comprising a soluble catalytic sector F_1 and a transmembrane sector F_0 functioning as proton channel. In organisms such as Escherichia coli and Bacillus subtilis, F_1 is composed of five subunits of α, β,

γ, δ and ε, while F_0 is composed of three subunits of a, b and c [Annu. Rev. Biochem., 66, 717 (1997)].

Concerning the F_0F_1-ATPase gene, the gene was isolated from Escherichia coli [Biochem. J., 224, 799 (1984)], Bacillus subtilis [J. Bacteriol., 176, 6802 (1994)], Bacillus megaterium [J. Biol. Chem., 264, 1528 (1989)], Bacillus firmus [Mol. Gen. Genet., 229, 292 (1991)], Bacillus sp. PS3 [Biochim. Biophys. Acta, 933, 141 (1988)], Methanosarcina barkeri [Biochem. Biophys. Res. Commun., 241, 427 (1997)], Lactobacillus acidophilus [Mol. Microbiol., 33, 1152 (1999)], Rhodobacter capsulatus [J. Bacteriol., 180, 416 (1998), Arch. Microbiol., 170, 385 (1998)] and the like, but no gene derived from microorganisms belonging to the genus Corynebacterium has been isolated yet.

Regarding the production of nucleoside 5'-triphosphate, methods using microorganisms (Japanese Published Unexamined Patent Application No.107593/1979; Japanese Published Unexamined Patent Application No. 51799/1984; J. Ferment. Bioeng., 68, 417 (1989)) and a method using enzymes (WO 98/22614) have been known. However, the productivity of nucleoside 5'-triphosphate is insufficient.

SUMMARY OF THE INVENTION

It is the purpose of the present invention to provide a protein complex having the F_0F_1-ATPase activity; a DNA

encoding the protein complex; a method for producing the protein complex having the F_0F_1-ATPase activity, using the DNA; and a method for producing nucleoside 5'-triphosphate, using the protein complex.

For this purpose, the present inventors have made various investigations. Consequently, the inventors have successfully isolated the genes encoding component proteins of which a protein complex having the F_0F_1-ATPase activity is composed, from Corynebacterium ammoniagenes. Thus, the present invention has been accomplished.

The present invention relates to the following (1) to (32) subject matters.

(1) A protein selected from the group consisting of the following proteins (a) to (c):

(a) a protein having the amino acid sequence represented by SEQ ID NO: 1;

(b) a protein having a modified one of the amino acid sequence represented by SEQ ID NO: 1, where one or more amino acids are deleted, substituted or added, and exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 2 to 8; and

(c) a protein having an amino acid sequence having 70 % or more identical to the amino acid sequence represented by SEQ ID NO: 1 and exerting the F_0F_1-ATPase activity when the protein

forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 2 to 8.

(2) A protein selected from the group consisting of the following proteins (a) to (c):

(a) a protein having the amino acid sequence represented by SEQ ID NO: 2;

(b) a protein having a modified one of the amino acid sequence represented by SEQ ID NO: 2, where one or more amino acids are deleted, substituted or added, and exreting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NO: 1 and SEQ ID NO: 3 to 8; and

(c) a protein having an amino acid sequence having 70 % or more identical to the amino acid sequence represented by SEQ ID NO: 2 and exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NO: 1 and SEQ ID NOS: 3 to 8.

(3) A protein selected from the group consisting of the following proteins (a) to (c):

(a) a protein having the amino acid sequence represented by SEQ ID NO: 3;

(b) a protein having a modified one of the amino acid sequence

represented by SEQ ID NO: 3, where one or more amino acids are deleted, substituted or added, and exerting the F_0F_1-ATPase activity when the protein forms a complex with all the proteins having the individual amino acid sequences represented by each of SEQ ID NOS: 1 and 2 and SEQ ID NOS: 4 to 8; and

(c) a protein having an amino acid sequence having 70 % or more identical to the amino acid sequence represented by SEQ ID NO: 3 and exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 and 2 and SEQ ID NOS: 4 to 8.

(4) A protein selected from the group consisting of the following proteins (a) to (c):

(a) a protein having the amino acid sequence represented by SEQ ID NO: 4;

(b) a protein having a modified one of the amino acid sequence represented by SEQ ID NO: 4, where one or more amino acids are deleted, substituted or added, and exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 3 and SEQ ID NOS: 5 to 8; and

(c) a protein having an amino acid sequence having 70 % or more identical to the amino acid sequence represented by SEQ ID NO: 4 and exerting the F_0F_1-ATPase activity when the protein

forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NO: 1 to 3 and SEQ ID NOS: 5 to 8.

(5) A protein selected from the group consisting of the following proteins (a) to (c):

(a) a protein having the amino acid sequence represented by SEQ ID NO: 5;

(b) a protein comprising a modified one of the amino acid sequence represented by SEQ ID NO: 5, where one or more amino acids are deleted, substituted or added, and exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 4 and SEQ ID NOS: 6 to 8; and

(c) a protein having an amino acid sequence having 70 % or more identical to the amino acid sequence represented by SEQ ID NO: 5 and exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 4 and SEQ ID NOS: 6 to 8.

(6) A protein selected from the group consisting of the following proteins (a) to (c):

(a) a protein having the amino acid sequence represented by SEQ ID NO: 6;

(b) a protein having a modified one of the amino acid sequence

represented by SEQ ID NO: 6, where one or more amino acids are deleted, substituted or added, and which can exert the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 5 and SEQ ID NOS: 7 and 8; and

(c) a protein having an amino acid sequence having 70 % or more identical to the amino acid sequence represented by SEQ ID NO: 6 and exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 5 and SEQ ID NOS: 7 and 8.

(7) A protein selected from the group consisting of the following proteins(a) to (c):

(a) a protein having the amino acid sequence represented by SEQ ID NO: 7;

(b) a protein having a modified one of the amino acid sequence represented by SEQ ID NO: 7, where one or more amino acids are deleted, substituted or added, and exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 6 and SEQ ID NO: 8; and

(c) a protein having an amino acid sequence having 70 % or more identical to the amino acid sequence represented by SEQ

ID NO: 7 and exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 6 and SEQ ID NO: 8.

(8) A protein selected from the group consisting of the following proteins (a) to (c):

(a) a protein having the amino acid sequence represented by SEQ ID NO: 8;

(b) a protein having a modified one of the amino acid sequence represented by SEQ ID NO: 8, where one or more amino acids are deleted, substituted or added, and exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 7; and

(c) a protein having an amino acid sequence having 70 % or more identical to the amino acid sequence represented by SEQ ID NO: 8 and exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins comprising the amino acid sequences represented by each of SEQ ID NOS: 1 to 7.

(9) A protein complex comprising eight proteins respectively selected from the eight groups as defined by each of (1) to (8).

(10) A DNA encoding any one of the proteins of (1) to (8).

(11) A DNA selected from the group consisting of the following

DNAs (a) and (b):

(a) a DNA having the nucleotide sequence represented by SEQ ID NO:9; and

(b) a DNA hybridizing with the DNA under stringent conditions and encoding a protein exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 2 to 8.

(12) A DNA selected from the group consisting of the following DNAs (a) and (b):

(a) a DNA having the nucleotide sequence represented by SEQ ID NO: 10; and

(b) a DNA hybridizing with the DNA under stringent conditions and encoding a protein exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NO: 1 and SEQ ID NOS: 3 to 8.

(13) A DNA selected from the group consisting of the following DNAs (a) and (b):

(a) a DNA having the nucleotide sequence represented by SEQ ID NO: 11; and

(b) a DNA hybridizing with the DNA under stringent conditions and encoding a protein exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ

ID NOS: 1 and 2 and SEQ ID NOS: 4 to 8.

(14) A DNA selected from the group consisting of the following DNAs (a) and (b):

(a) a DNA having the nucleotide sequence represented by SEQ ID NO:12; and

(b) a DNA hybridizing with the DNA under stringent conditions and encoding a protein exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the individual amino acid sequences represented by each of SEQ ID NOS: 1 to 3 and SEQ ID NOS: 5 to 8.

(15) A DNA selected from the group consisting of the following DNAs (a) and (b):

(a) a DNA having the nucleotide sequence represented by SEQ ID NO:13; and

(b) a DNA hybridizing with the DNA under stringent conditions and encoding a protein exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 4 and SEQ ID NOS: 6 to 8.

(16) A DNA selected from the group consisting of the following DNAs (a) and (b):

(a) a DNA having the nucleotide sequence represented by SEQ ID NO: 14; and

(b) a DNA hybridizing with the DNA under stringent conditions and encoding a protein exerting the F_0F_1-ATPase activity when

the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 5 and SEQ ID NOS: 7 and 8.

(17) A DNA selected from the group consisting of the following DNAs (a) and (b):

(a) a DNA having the nucleotide sequence represented by SEQ ID NO: 15; and

(b) a DNA hybridizing with the DNA under stringent conditions and encoding a protein exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 6 and SEQ ID NO: 8.

(18) A DNA selected from the group consisting of the following DNAs (a) and (b):

(a) a DNA having the nucleotide sequence represented by SEQ ID NO: 16; and

(b) a DNA hybridizing with the DNA under stringent conditions and encoding a protein exerting the F_0F_1-ATPase activity when the protein forms a complex with all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 7.

(19) A DNA comprising the eight DNAs respectively selected from the eight groups as defined by each of (11) to (18).

(20) A DNA having the nucleotide sequences represented by SEQ ID NOS: 9 to 16.

(21) A DNA having the nucleotide sequence represented by SEQ ID NO: 21.

(22) The DNA according to any one of (10) to (21), where the DNA is derived from a microorganism belonging to the genus Corynebacterium.

(23) The DNA according to any one of (10) to (21), where the DNA is derived from a microorganism of the species Corynebacterium ammoniagenes.

(24) A recombinant DNA constructed by inserting the DNA according to any one of (10) to (18) into a vector.

(25) A recombinant DNA constructed by inserting the DNA according to any one of (19) to (21) into a vector.

(26) A transformant obtained by transformation of a host cell with the recombinant DNA of (24) or (25).

(27) A transformant of (26), where the host cell is a microorganism of the species Escherichia coli, Corynebacterium glutamicum or Corynebacterium ammoniagenes.

(28) A method for producing a protein of any one of (1) to (8), which comprises culturing a transformant obtained by transformation of a host cell with the recombinant DNA of (24) in a culture medium, so as to allow the protein of any one of (1) to (8) to be expressed and accumulated in the culture and harvesting the protein from the culture.

(29) A method for producing a protein complex having the F_0F_1-ATPase activity, which comprises culturing a transformant

obtained by transformation of a host cell with the recombinant DNA of (25) in a culture medium, so as to allow a protein complex having the F_0F_1-ATPase activity to be expressed and accumulated in the culture and recovering the protein complex from the culture.

(30) A method for producing nucleoside 5'-triphosphate, which comprises by use of a culture of a transformant obtained by transformation of a host cell with the recombinant DNA of (25) or a treated product of the culture as an enzyme source, allowing the enzyme source and a precursor of nucleoside 5'-triphosphate to co-exist with each other in an aqueous medium to generate and accumulate the nucleoside 5'-triphosphate and recovering the nucleoside 5'-triphosphate from the aqueous medium.

(31) The method of (30), where the precursor of nucleoside 5'-triphosphate is adenine, guanine, uracil, cytosine, hypoxanthine, adenosine, guanosine, uridine, cytidine, inosine, adenosine 5'-monophosphate, guanosine 5'-monophosphate, uridine 5'-monophosphate, cytidine 5'-monophosphate or inosine 5'-monophosphate.

(32) The method of (30), where the nucleoside 5'-triphosphate is adenosine 5'-triphosphate, guanosine 5'-triphosphate, uridine 5'-triphosphate or cytidine 5'-triphosphate.

BRIEF DESCRIPTION OF THE DRAWING

Fig.1 shows restriction maps of fragments inserted in plasmids pUH71, pE61 and pDW31 and the open reading frames contained in the fragments.

DETAILED DESCRIPTION OF THE INVENTION

(1) Preparation of the DNA of the present invention

(a) Preparation of a DNA library

The protein complex of the present invention is a protein complex comprising the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 8 as components.

As long as the protein complex exerts the F_0F_1-ATPase activity, one or more amino acids can be deleted, substituted or added in the amino acid sequences of the individual proteins.

The protein having an amino acid sequence with one or more amino acids deleted, substitutied or added, which can be the component of the protein complex having the F_0F_1-ATPase activity, can be obtained by introducing mutation in the DNA encoding the protein having any one of SEQ ID NOS: 1 to 8, via the site-directed mutagenesis described in Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press (1989) (abbreviated as Molecular Cloning, Second edition, hereinafter), Current Protocols in Molecular Biology, John Wiley & Sons (1987-1997) (abbreviated Current Protocols in Molecular Biology, hereinafter), Nucleic

Acids Research, 10, 6487 (1982), Proc. Natl. Acad. Sci. USA, 79, 6409(1982), Gene, 34, 315 (1985), Nucleic Acids Research, 13, 4431 (1985), Proc. Natl. Acad. Sci. USA, 82, 488 (1985), and the like.

The number of the amino acids to be deleted, substituted, or added is not particularly limited, but should be such that deletion, substitution or addition according to well-known methods such as site-directed mutagenesis can be ocurred. The number is one to several tens, preferably one to 20, more preferably one to 10, still more preferably one to 5.

In order for the protein complex according to the present invention to have the F_0F_1-ATPase activity, each component protein of the protein complex has such identity to the corresponding amino acid sequences represented by each of SEQ ID NOS: 1 to 8, as at least 60 %, preferably 80 % or more, more preferably 95 % or more. The identity of a nucleotide sequence or an amino acid sequence can be determined using the algorithm "BLAST" by Karlin and Altschl [Proc. Natl. Acad. Sci. USA, 90, 5873-5877 (1993)]. The programs called "BLASTN" and "BLASTX" have been developed based on the above algorithm [J. Mol. Biol., 215, 403-410 (1990)]. In the case of analyzing a nucleotide sequence based on BLAST, the parameter can be set to e.g. score=100, wordlength=12. In the case of analyzing an amino acid sequence based on BLASTX, the parameter can be set to e.g. score=50, wordlength=3. In

the case of using BLAST or Gapped BLAST program, a default parameter of each program can be used. The specific analysis methods of using the above programs are known in the art (http://www.ncbi.nlm.nih.gov.).

However, the protein of the present invention does not include any proteins having the amino acid sequences in the public domain.

The DNAs of the present invention encode the proteins of the present invention or the protein complex comprising the proteins as the components and can be isolated from a microorganism of the genus Corynebacterium. The microorganism belonging to the genus Corynebacterium includes, for example, Corynebacterium ammoniagenes, specifically Corynebacterium ammoniagenes strain ATCC6872. Specific examples of the DNA of the present invention include DNAs having the nucleotide sequence represented by any one of SEQ ID NOS: 9 to 16, a DNA comprising all the individual nucleotide sequences, and a DNA having the nucleotide sequence represented by SEQ ID NO: 21.

Additionally, DNAs hybridizing under stringent conditions with the DNA having the nucleotide sequence represented by any one of SEQ ID NOS: 9 to 16, a DNA comprising all the individual nucleotide sequences, and a DNA represented by the nucleotide sequence represented by SEQ ID NO: 21 are also encompassed within the scope of the DNA of the present

invention. The DNA hybridizing under stringent conditions can be isolated by colony hybridization, plaque hybridization or Southern hybridization or the like, using the DNAs of the nucleotide sequences represented by any one of SEQ ID NOS: 9 to 16, the DNA comprising all the individual nucleotide sequences or the DNA of the nucleotide sequence represented by SEQ ID NO: 21 as the probe. Specifically, the DNA includes a DNA isolated and identified by using a filter on which the colony- or plaque-derived DNA is immobilized, for hybridization in the presence of 0.7 to 1.0 mol/liter NaCl at 65 °C and subsequent washing of the filter with 0.1 × to 2 × SSC (saline-sodium citrate) solution [1 × SSC solution (150 mmol/liter NaCl, 15 mmol/liter sodium citrate); n × means solution at n-fold concentration] under a condition of 65 °C.

The hybridization can be promoted according to the method described in experimental text books, such as Molecular Cloning, Second edition; and Current Protocols in Molecular Biology, DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University (1995). Specifically, the hybridizable DNA includes DNA with at least 80 %, preferably 95 % or more identical to the DNA of the nucleotide sequence represented by any one of the nucleotide sequences of SEQ ID NOS: 9 to 16, the DNA comprising all the individual nucleotide sequences, or the DNA of the nucleotide sequence represented

by SEQ ID NO: 21, when the identity is calculated by BLAST and the like as described above.

However, the DNA of the present invention does not include DNAs in the public domain.

The method for isolating the DNA of the present invention is described hereinbelow.

A microorganism belonging to the genus Corynebacterium is cultured according to a known method [for example, Appl. Microbiol. Biotechnol., 39, 318 (1993)]. After culturing, the chromosomal DNA of the microorganism is isolated and purified according to a known method [for example, Current Protocols in Molecular Biology, Agric. Biol. Chem., 49, 2925 (1985)].

A method for preparing a DNA library includes methods described in, for example, Molecular Cloning, Second edition; and Current Protocols in Molecular Biology, DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University Press (1995).

As the cloning vector for preparing the DNA library, any cloning vector autonomously replicable in Escherichia coli strain K12 can be used, including phage vector and plasmid vector. Specifically, the cloning vector includes ZAP Express [manufactured by Stratagene, Strategies, 5, 58 (1992)], λzap II (manufactured by Staratagene), λgt10 and λgt11 [DNA Cloning, A Practical Approach, 1, 49 (1985)], λTriplEx

(manufactured by Clontech), λExCell (manufactured by Amersham Pharmacia Biotech), pBluescript II KS (-) and pBluescript II SK (+) [manufactured by Stratagene, Nucleic Acids Research, 17, 9494 (1989)], pUC18 [Gene, 33, 103 (1985)] and the like.

As the Escherichia coli for transformation with the vector in which the DNA is inserted, any microorganism belonging to the species Escherichia coli can be used. Specifically, the microorganism includes Escherichia coli XL1-Blue MRF' [manufactured by Stratagene, Strategies, 5, 81 (1992)], Escherichia coli C600 [Genetics, 39, 440 (1954)], Escherichia coli Y1088 [Science, 222, 778 (1983)], Escherichia coli Y1090 [Science, 222, 778 (1983)], Escherichia coli NM522 [J. Mol. Biol., 166, 1 (1983)], Escherichia coli K802 [J. Mol. Biol., 16, 118 (1966)], Escherichia coli JM109 [Gene, 33, 103 (1985)] and the like.

(b) Acquisition of the DNA of the present invention

The objective clone can be selected from the DNA library by colony hybridization, plaque hybridization or Southern hybridization , as described in experimental textbooks such as Molecular Cloning, Second edition; and Current Protocols in Molecular Biology, DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University (1995).

The DNA probe for use in the hybridization includes, for example, DNA isolated by PCR [PCR Protocols, Academic Press (1990)] using DNA primers designed from known sequences,

in addition to known genes or parts of the known genes and DNA synthesized on the basis of known sequences. The DNA probe includes, for example, a DNA fragment isolated from Escherichia coli chromosome by using the synthetic DNAs of SEQ ID NOS: 17 and 18 as primers, designed on the basis of the sequence of F_0F_1-ATPase β subunit gene of Escherichia coli.

The isolated DNA as it is or after cleavage with an appropriate restriction endonuclease is inserted into a vector. Then, the nucleotide sequence of the DNA is determined by methods for nucleotide sequencing for general use, for example the dideoxy method [Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)] using 373A·DNA sequencer (manufactured by Perkin Elmer).

In case that the resulting DNA contains only a part of the DNA of the present invention, the full-length DNA can be isolated by hybridization with a DNA fragment as probe, isolated by PCR using primers designed from the isolated DNA sequence.

The vector in which the isolated DNA of the present invention is inserted includes pBluescript KS (-) (manufactured by Stratagene), pDIRECT [Nucleic Acids Research, 18, 6069 (1990)], pCR-Script Amp SK (+) (manufactured by Staratagene), pT7Blue (manufactured by Novagen), pCR II (manufactured by Invitrogen Corporation), pCR-TRAP (manufactured by Gene Hunter) and pNoTAT7 (manufactured by 5 Prime → 3 Prime Co.).

The DNA having the novel nucleotide sequence isolated as described above includes, for example, the DNA having the nucleotide sequence represented by SEQ ID NO: 21.

The DNA having the nucleotide sequence represented by SEQ ID NO: 21 encodes all the individual proteins having the amino acid sequences represented by each of SEQ ID NOS: 1 to 8.

The bacterial strain carrying a plasmid comprising the DNA having the nucleotide sequence represented by SEQ ID NO: 21 includes, for example, Escherichia coli JM109/pE61, JM109/pDW31 and JM109/pUH71.

Further, the objective DNA can be isolated by preparing primers based on the nucleotide sequence thus determined and carrying out PCR using the chromosomal DNA as the template and the primers.

The DNA encoding any one of the component proteins of the protein complex of the present invention can be obtained by cleaving the DNA obtained above with a restriction endonuclease and the like.

For example, the DNA encoding any one of the proteins of SEQ ID NOS: 1 to 8 can be isolated as the DNA having any one of the nucleotide sequences repersented by SEQ ID NOS: 9 to 16, respectively, by cleaving the DNA of SEQ ID NO: 21 and individually isolating the resulting DNA.

Based on the thus determined nucleotide sequence of

DNA, furthermore, the objective DNA can be prepared by chemical synthesis by means of DNA synthesizers such as the DNA synthesizer of Model 8905, manufactured by Perceptive Biosystems, Co.

[2] Production of the proteins and the protein complex of the present invention

The proteins and the protein complex of the present invention can be prepared, for example, by expressing the DNA of the present invention in a host cell by the following procedures according to the method described in Molecular Cloning, Second edition, Current Protocols in Molecular Biology and the like.

More specifically, a recombinant DNA is prepared by inserting the DNA of the present invention downstream the promoter of an appropriate expression vector, which is then used for transformation of a host cell compatible with the expression vector, so that a transformant in which the protein or protein complex of the present invention is expressed can be obtained. As the host cell, any host cell capable of having the objective gene expressed, such as bacteria, yeast, an animal cell, an insect cell and a plant cell, can be used. As the expression vector, a vector having autonomous replication in the host cell or integration into the chromosome of the host cell and containing a promoter at a position where

the DNA encoding the protein or protein complex of the present invention can be transcribed, is used.

In case that prokaryotic organisms such as bacteria are used as host cells, it is preferred that the recombinant DNA containing the DNA encoding the protein or protein complex of the present invention can autonomously replicate in the bacteria and simultaneously that, the recombinant DNA is a vector composed of promoter, ribosome binding sequence, the DNA encoding the protein or protein complex of the present invention and a transcription termination sequence. The recombinant DNA may contain a gene regulating the promoter.

The expression vector includes, for example, pHelix1 (manufactured by Roche Diagnostics), pKK233-2 (manufactured by Amersham Pharmacia Biotech), pSE280 (manufactured by Invitrogen Corporation), pGEMEX-1 (manufactured by Promega Corporation), pQE-8 (manufactured by Qiagen), pKYP10 (Japanese Published Unexamined Patent Application No.110600/1983, USP4686191), pKYP200 [Agric. Biol. Chem., 48, 669 (1984)], pLSA1 [Agric. Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)], pBluescript II KS(-) (manufactured by Stratagene), pTrs30 [prepared from Escherichia coli JM109/pTrS30 (FERM BP-5407)], pTrs32 [prepared from Escherichia coli JM109/pTrS32 (FERM BP-5408)], pGHA2 [prepared from Escherichia coli IGHA2 (FERM B-400), Japanese Published Unexamined Patent Application

No.221091/1985, USP4868125], pGKA2 [prepared from Escherichia coli IGKA2 (FERM BP-6798), Japanese Published Unexamined Patent Application No.221091/1985, USP4868125], pTerm2 (USP4686191, USP4939094, USP5160735), pSupex, pUB110, pTP5, pC194, pEG400 [J. Bacteriol., 172, 2392 (1990)], pGEX (manufactured by Amersham Pharmacia Biotech), pET system (manufactured by Novagen) and pSupex. Additionally, expression vectors with autonomous replication potencies in microorganisms of genus Corynebacterium include for example pCG1 (Japanese Published Unexamined Patent Application No.134500/1983); pCG2 (Japanese Published Unexamined Patent Application No.35197/1983); pCG4 and pCG11 (both in Japanese Published Unexamined Patent Application No.183799/1982, USP4500640); pCE54 and pCB101 (both in Japanese Published Unexamined Patent Application No.105999/1983, USP4710471); pCE51, pCE52 and pCE53 [all in Mol. Gen. Genet., 196, 175 (1984)]; pAJ1844 (Japanese Published Unexamined Patent Application No.21614/1983, USP4514502); pHK4 (Japanese Published Unexamined Patent Application No.20399/1995, USP5616480); pHM1519 [Agric. Biol. Chem., 48, 2901, (1985)]; pCV35 and pECM1 [both in J. Bacteriol., 172, 1663 (1990)]; pC2 [Plasmid, 36, 62 (1996)] and the like.

As the promoter, any promoter from which a gene can be expressed in the host cell can be used. For example, promoters derived from Escherichia coli and phages, such as

trp promoter (P_{trp}), lac promoter, P_L promoter, P_R promoter and T7 promoter are mentioned. Additionally, artificially designed and modified promoters can also be used, such as two aligned P_{trp} promoters in series (P_{trp}×2), tac promoter, lacT7 promoter, and let I promoter. Still additionally, the promoter capable of functioning in bacteria of the genus Corynebacterium [Microbiology, 142, 1297 (1996), Appl. Microbiol. Biotechnol., 53, 674 (2000)] and the like can also be used.

A plasmid with the distance between the ribosome binding sequence, namely the Shine-Dalgarno sequence and the initiation codon as adjusted to an appropriate length (for example, 6 to 18 bases) is preferably used.

By modifying the nucleotide sequence of the region encoding the protein or protein complex of the present invention to make codons optimum for the expression in hosts, the productivity of the objective protein or protein complex can be improved.

For the recombinant vector of the present invention, a transcription termination sequence is not necessarily required for the expression of the DNA of the present invention. However, it is preferred that a transcription termination sequence is arranged immediately downstream the structural gene.

The host cell includes microorganisms of the genera

Escherichia, Serratia, Bacillus, Brevibacterium, Corynebacterium, Microbacterium and Pseudomonas, for example, Escherichia coli XL1-Blue, Escherichia coli XL2-Blue, Escherichia coli DH1, Escherichia coli MC1000, Escherichia coli KY3276, Escherichia coli W1485, Escherichia coli JM109, Escherichia coli HB101, Escherichia coli No.49, Escherichia coli W3110, Escherichia coli NY49, Serratia ficaria, Serratia fonticola, Serratia liquefaciens, Serratia marcescens, Bacillus subtilis, Bacillus amyloliquefacines, Brevibacterium immariophilum ATCC14068, Brevibacterium saccharolyticum ATCC14066, Brevibacterium flavum ATCC14067, Brevibacterium lactofermentum ATCC13869, Corynebacterium ammoniagenes ATCC6872, Corynebacterium ammoniagenes ATCC21170, Corynebacterium glutamicum ATCC13032, Corynebacterium acetoacidophilum ATCC13870, Microbacterium ammoniaphilum ATCC15354 and Pseudomonas sp. D-0110.

As the method for transformation with the recombinant DNA, any method to introduce DNA into the host cell can be used, including, for example, the method using calcium ion [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)], protoplast method [Japanese Published Unexamined Patent Application No.248394/1988, Japanese Published Unexamined Patent Application No.186492/1982, USP4683205, Japanese Published Unexamined Patent Application No.56678/1983, USP4681847, J. Bacteriol., 159, 306 (1984)], electroporation method

(Japanese Published Unexamined Patent Application No.207791/1990) or the methods described in Gene, 17, 107 (1982) and Mol. Gen. Genet., 168, 111 (1979) are mentioned. Otherwise, a DNA library of the chromosome of a microorganism of the genus Corynebacterium is prepared by using Escherichia coli, then DNA is transferred from Escherichia coli to a microorganism of the genus Corynebacterium via conjugation according to known methods [J. Bacteriol. 172, 1663 (1990), J. Bacteriol. 178, 5768 (1996)].

In case that yeast is used as the host cell, the expression vector includes, for example, YEp13 (ATCC37115), YEp24 (ATCC37051) and YCp50 (ATCC37419).

As the promoter, any promoter from which a gene can be expressed in yeast strains can be used, including, for example, the promoter of the gene of the glycolytic pathway such as hexokinase, PHO5 promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat shock protein promoter, MFα1 promoter and CUP 1 promoter.

The host cell includes, for example, microorganisms of the genus Saccharomyces, Kluyveromyces, Trichosporon or Schwanniomyces, for example, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Trichosporon pullulans and Schwanniomyces alluvius.

As the method for transformation with the recombinant DNA, any method to introduce DNA into yeast can be used,

including, for example, electroporation method [Methods. Enzymol., 194, 182 (1990)], spheroplast method [Proc. Natl. Acad. Sci. USA, 84, 1929 (1978)], lithium acetate method [J. Bacteriology, 153, 163 (1983)], and the method described in Proc. Natl. Acad. Sci. USA, 75, 1929 (1978).

In case that an animal cell is used as the host cell, the expression vector includes, for example, pcDNAI, pcDM8 (commercially available from Funakoshi Co., Ltd.), pAGE107 [Japanese Published Unexamined Patent Application No.22979/1991, USP516735; Cytotechnology, 3, 133, (1990)], pAS3-3 (Japanese Published Unexamined Patent Application No.227075/1990, USP5218092), pCDM8 [Nature, 329, 840, (1987)], pcDNAI/Amp (manufactured by Invitrogen Corporation), pREP4 (manufactured by Invitrogen Corporation), pAGE103 [J. Biochemistry, 101, 1307 (1987)] and pAGE210.

As the promoter, any promoter from which a gene can be expressed in animal cells can be used, including, for example, the promoter of the immediate early (IE) gene of cytomegalovirus (CMV), the SV40 early promoter , the promoter of retrovirus, metallothionein promoter, heat shock promoter and SRα promoter. Additionally, an enhancer of the IE gene of human CMV can be used together with such promoters.

The host cell includes Namalwa cell as a human cell, COS cell as a monkey cell, CHO cell as Chinese hamster cell, HBT5637 (Japanese Published Unexamined Patent Application

No.299/1988, ATCC No. ATB-9) and the like.

As the method for transformation with the recombinant vector, any method to introduce DNA into animal cells can be used, including, for example, electroporation method [Cytotechnology, 3, 133 (1990)], calcium phosphate method (Japanese Published Unexamined Patent Application No.227075/1990, USP5218092) and lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)].

In case that insect cells are used as the host cells, the protein or protein complex of the present invention can be expressed by methods described in, for example, Current Protocols in Molecular Biology, Baculovirus Expression Vectors, A Laboratory Manual, W. H. Freeman and Company, New York (1992) and Bio/Technology, 6, 47 (1988).

Specifically, a recombinant virus is recovered in the culture supernatant of an insect cell via co-transfection of the insect cell with the recombinant gene transfer vector and baculovirus; further, an insect cell is infected with the recombinant virus, to express the protein or protein complex of the present invention.

The gene transfer vector for use in the method includes, for example, pVL1392, pVL1393 and pBlueBacIII (all manufactured by Invitrogen Corporation).

As the baculovirus, for example, Autographa californica nuclear polyhedrosis virus can be used as a virus

which infects insects of the family Noctuidae.

As the insect cells, Sf9 and Sf21 as the ovarian cells of Spodoptera frugiperda [Baculovirus Expression Vectors, A Laboratory Manual, W. H. Freeman and Company, New York (1992)] and High 5 as the ovarian cell of Trichoplusia ni (manufactured by Invitrogen Corporation) can be used.

The method for the co-transfection of the insect cells with the recombinant gene transfer vector and the baculovirus for the preparation of the recombinant virus, includes, for example, the calcium phosphate method (Japanese Published Unexamined Patent Application No.227075/1990, USP5218092) and the lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)].

In case that plant cells are used as the host cells, the expression vector includes, for example, Ti plasmid and tobacco mosaic virus vector.

As the promoter, any promoter including, for example, the 35S promoter of cauliflower mosaic virus (CaMV) and rice actin 1 promoter can be used, as long as a gene can be expressed from the promoter in plant cells.

The host cell includes plant cells of, for example, tobacco, potato, tomato, carrot, soybean, rape, alfalfa, rice, wheat, and barley.

As the method for transformation with the recombinant DNA, any method for introducing DNA into plant cells can be

used, including, for example, the method using Agrobacterium (Japanese Published Unexamined Patent Application No.140885/1984, Japanese Published Unexamined Patent Application No. 70080/1985 and WO 94/00977), the electroporation method (Japanese Published Unexamined Patent Application No.251887/1985) and the method using particle gun (Japanese Patent No. 2606856 and Japanese Patent No. 2517813).

As the method for expressing the gene, secretory expression, or fusion protein expression or the like can be carried out according to the method described in Molecular Cloning, Second edition, in addition to direct expression.

In case of the expression in yeast, animal cells, insect cells or plant cells, a protein or protein complex with addition of sugar or sugar chain can be recovered.

The protein or protein complex of the present invention can be produced by culturing the transformant thus constructed in a culture medium to allow the transformant to express and accumulate the protein or protein complex of the present invention in the culture and recovering the protein or protein complex from the culture. Culturing the transformant of the present invention in a culture medium can be carried out according to a usual method to be applied for in culturing hosts.

As the culture medium for culturing the transformant

obtained by using bacteria such as Escherichia coli or eukaryotic organisms such as yeast as the hosts, any of natural and synthetic culture media containing carbon sources, nitrogen sources, inorganic salts and the like, which can be assimilated by the biological organism and in which the transformant can be cultured efficiently can be used.

As the carbon source, any carbon source assimilated by the biological organism can be used, such as carbohydrates such as glucose, fructose, sucrose, molasses containing these substances, starch or starch hydrolyzates; organic acids such as acetic acid and propionic acid; and alcohols such as ethanol and propanol.

As the nitrogen source, ammonia, ammonium salts of inorganic acids or organic acids, such as ammonium chloride, ammonium sulfate, ammonium acetate and ammonium phosphate; other nitrogen-containing compounds; and peptone, meat extract, yeast extract, corn steep liquor, casein hydrolyzates, soy bean bran, soy bean bran hydrolyzates, various fermenting bacteria and digested products thereof and the like can be used.

As the inorganic salts, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate and calcium carbonate and the like can be used.

The culturing is generally carried out under aerobic conditions, by shaking culture or submerged aeration agitation culture. The culturing temperature is preferably 15 to 40 °C, and the culturing period is generally 16 hours to 7 days. The pH during the culturing is retained at 3.0 to 9.0. The pH is adjusted with inorganic or organic acids, alkali solutions, urea, calcium carbonate, ammonia and the like.

Additionally, antibiotics such as ampicillin and tetracycline can be added to the culture medium if required.

For culturing a microorganism transformed with a recombinant vector where an inducible promoter is used as promoter, an inducer can be added if required. For example, for culturing a microorganism transformed with a recombinant vector where lac promoter is used, isopropyl-β-D-thiogalactopyranoside and the like can be added to the culture medium, and for culturing a microorganism transformed with a recombinant vector where trp promoter is used, indole acrylic acid and the like can be added to the culture medium.

As the culture medium for culturing a transformant obtained by using an animal cell as the host, culture media for general use, such as RPMI 1640 medium [The Journal of the American Medical Association, 199, 519 (1967)], Eagle's MEM [Science, 122, 501 (1952)], Dulbecco's modified MEM [Virology, 8, 396 (1959)], 199 medium [Proceeding of the

Society for the Biological Medicine, 73, 1 (1950)] or culture media prepared by adding fetal calf serum to these culture media can be used.

The culturing is generally carried out under conditions, such as at pH 6 to 8 and 30 to 40 °C in the presence of 5 % CO_2 for one to 7 days.

Additionally, antibiotics such as kanamycin and penicillin can be added to the culture medium during the culturing if required.

As the culture medium for culturing a transformant obtained by using an insect cell as the host, TNM-FH medium (manufactured by Pharmingen); Sf-900 II SFM (manufactured by Life Technologies); ExCell 400 and ExCell 405 (both manufactured by JRH Biosciences, Co.); Grace's Insect Medium [Nature, 195, 788 (1962)]; and the like can be used.

The culturing is generally conducted under conditions, for example at pH 6 to 7 and 25 to 30 °C for one to 5 days.

Additionally, antibiotics such as gentamycin can be added to the culture medium if reqired.

A transformant obtained by using a plant cell as the host can be cultured as a cell or after differentiation into a differentiated plant cell or a plant organ. As the culture medium for culturing the transformant, Murashige and Skoog (MS) medium, White's medium, or culture media prepared by adding plant hormones such as auxin and cytokinin or the like

to these culture media can be used.

The culturing is generally conducted under conditions, for example at pH 5 to 9 and 20 to 40 °C for 3 to 60 days.

Additionally, antibiotics such as kanamycin and hygromycin can be added to the culture medium if required.

As described above, the protein or protein complex of the present invention can be produced, by culturing a microorganism-, animal cell- or plant cell-derived transformant carrying the recombinant DNA into which the DNA encoding the protein or protein complex of the present invention is inserted by general culturing methods, to allow the transformant to express and accumulate the protein or protein complex and recovering the protein or protein complex from the culture.

As the method for expressing the gene, secretory expression or expression as a fusion protein can be carried out according to the method described in Molecular Cloning, Second edition, in addition to direct expression.

The method for expressing the protein or protein complex of the present invention includes a method for expressing inside host cells, a method for secreting outside host cells, and a method for expressing on the outer membrane of host cells. By changing a host cell to be used or modifying the structure of the protein to be expressed, the method can be selected.

In case that the protein or protein complex of the present invention is expressed inside the host cell or on the outer membrane of the host cell, the protein or protein complex can be secreted outside the host cell, according to the method of Paulson, et al. [J. Biol. Chem., 264, 17619 (1989)], the method of Law, et al. [Proc. Natl. Acad. Sci., USA, 86, 8227 (1989), Genes Develop., 4, 1288 (1990)], or the methods described in Japanese Published Unexamined Patent Application No.336963/1993, WO 94/23021 and the like.

Specifically, the protein or protein complex of the present invention can be secreted outside the host cell, by expressing the protein or protein complex of the present invention in a form such that signal peptide is added to a protein containing the active site of the protein or protein complex of the present invention, by means of the genetic engineering.

According to the method described in Japanese Published Unexamined Patent Application No.227075/1990 (USP5218092), the productivity can be improved by utilizing gene amplification systems using dihydrofolate reductase genes and the like. So as to isolate and purify the protein or protein complex of the present invention from the culture of the transformant, general methods for isolation and purification of enzymes can be used.

In case that the protein or protein complex of the

present invention is expressed, for example, in the soluble state inside a cell, the cell is recovered by centrifugation after culturing and is then suspended in an aqueous buffer, which is subsequently disrupted with an ultrasonicator, French press, Manton-Gaulin homogenizer, Dinomill and the like, to recover a cell-free extract. From the supernatant recovered by centrifuging the cell-free extract, a purified product sample can be isolated using known methods to isolate and purify enzymes, including, for example, solvent extraction, salting out with ammonium sulfate, desalting, precipitation with organic solvents, anion exchange chromatography using resins such as diethylaminoethyl (DEAE)-Sepharose, DIAION HPA-75 (manufactured by Mitsubishi Chemical Industry, Co.), cation exchange chromatography using resins such as S-Sepharose FF (manufactured by Amersham Pharmacia Biotech, Co.), hydrophobic chromatography using resins such as phenyl Sepharose, gel filtration method using molecular sieve, affinity chromatography, chromatofocusing, electrophoresis such as isoelectric focusing, singly or in combination.

In case that the protein or protein complex is expressed in the form of an inclusion body inside a cell, the cell is recovered and disrupted in the same manner as descrived above, followed by centrifugation to recover a precipitate fraction, from which the protein or protein complex is recovered by

known methods. The inclusion body of the protein or protein complex is solubilized with a protein denaturant. The protein-solubilized solution is diluted with or dialyzed to against a solution without protein denaturant or a solution where the concentration of the protein denaturant is at lower level not to denature the protein or protein complex, to refold the protein or protein complex in a normal tertiary structure. Subsequently, the resulting protein or protein complex is subjected to the same method for isolation and purification as described above, to isolate a purified product sample.

In case that the protein or protein complex of the present invention or derivatives thereof such as a glycosylated form are secreted extracellularly, the protein or protein complex or the derivatives thereof, such as a glycosylated form, can be recovered in the culture supernatant. More specifically, the culture is treated by the same means as described above, such as centrifugation, to recover the soluble fraction, from which a purified product sample can be isolated by the method for isolation and purification as described above.

[3] Production of nucleoside 5'-triphosphate

Using as an enzyme source the culture of the transformant prepared described above in [2] or a treated product of the culture, nucleoside 5'-triphosphate can be

produced by reacting a precursor of nucleoside 5'-triphosphate with the enzyme source in an aqueous medium.

The treated product of the culture includes, for example, a concentrate of the culture, a dried product of the culture, transformant cells recovered by centrifuging the culture, a dried product of the transformant cells, a freeze-dried product of the transformant cells, a detergent-treated product of the transformant cells, a sonicated product of the transformant cells, a mechanically disrupted product of the transformant cells, a solvent-treated product of the transformant cells, a enzyme-treated product of the transformant cells, a protein fraction of the transformant cells, an immobilized product of the transformant cells or an enzyme sample extracted from the transformant cells.

As the enzyme source for generating nucleoside 5'-triphosphate, the transformant cells are used at a concentration of 1 g/liter to 500 g/liter, preferably 10 g/liter to 300 g/liter based on wet weight.

The precursor of nucleoside 5'-triphosphate includes, for example, adenine, guanine, uracil, cytosine, hypoxanthine, adenosine, guanosine, uridine, cytidine, inosine, adenosine 5'-monophosphate, guanosine 5'-monophosphate, uridine 5'-monophosphate, cytidine 5'-monophosphate, and inosine 5'-monophosphate.

The nucleoside 5'-triphosphate includes for example

adenosine 5'-triphosphate, guanosine 5'-triphosphate, uridine 5'-triphosphate, and cytidine 5'-triphosphate.

The aqueous medium for use in generating nucleoside 5'-triphosphate includes, for example, water; buffers such as phosphate buffer, carbonate buffer, acetate buffer, borate buffer, citrate buffer and Tris buffer; alcohols such as methanol and ethanol; esters such as ethyl acetate; ketones such as acetone; and amides such as acetamide. Additionally, the liquid culture of the microorganism used as the enzyme source can also be used as the aqueous medium.

For the generation of nucleoside 5'-triphosphate, a detergent or an organic solvent can be added if required. As the detergent, any of detergents promoting the generation of nucleoside 5'-triphosphate can be used, including, for example, nonionic detergents such as polyoxyethylene octadecylamine (for example, Nymeen S-215, manufactured by Nippon Oil & Fats Co., Ltd.), cationic detergents such as cetyltrimethylammonium bromide and alkyldimethyl benzylammonium chloride (for example, Cation F2-40E, manufactured by Nippon Oil & Fats Co., Ltd.) and anionic detergents such as lauroyl sarcosinate and tertiary amines such as alkyldimethylamine (for example, tertiary amine FB, manufactured by Nippon Oil & Fats Co., Ltd.), singly or in combination of several types thereof. The detergent is generally used at a concentration of 0.1 to 50 g/liter. The

organic solvent includes, for example, xylene, toluene, aliphatic alcohol, acetone and ethyl acetate and is generally used at a concentration of 0.1 to 50 ml/liter.

The nucleoside 5'-triphosphate generating reaction is carried out in an aqueous medium under conditions of pH 5 to 10, preferably pH 6 to 8 and 20 to 60 °C, for one to 96 hours. For the generating reaction, inorganic salts such as magnesium chloride can be added if required.

The nucleoside 5'-triphosphate generated in an aqueous medium is determined by known methods (for example, WO 98/12343) using HPLC.

The nucleoside 5'-triphosphate generated in the reaction solution can be isolated by known methods using activated charcoal, ion exchange resins and the like.

The present invention is illustrated in the following examples, but the present invention is not limited to these examples.

Example 1

Preparation of the chromosomal DNA of Corynebacterium ammoniagenes strain ATCC6872

Corynebacterium ammoniagenes strain ATCC6872 was inoculated in 8 ml of a culture medium prepared by adding glycine (10 mg/ml) to CM medium (10 mg/ml polypeptone, 10 mg/ml meat extract, 5 mg/ml yeast extract, 3 mg/ml sodium

chloride, 30 μg/ml biotin, pH7.2), for culturing at 30 °C overnight.

After culturing, the cells were collected from the resulting culture via centrifugation.

The cells were washed with TE buffer [10 mmol/liter Tris-HCl, 1 mmol/liter ethylenediaminetetraacetic acid (EDTA), pH8.0] and subsequently suspended in 800 μl of the same buffer. To the suspension were added 40 μl of 50 mg/ml lysozyme solution and 20 μl of 10 mg/ml RNase A solution, and the resulting solution was incubated at 37 °C for one hour. To the resulting solution was added 20 μl of 20 % sodium dodecylsulfate (SDS) solution, and the resulting solution was incubated at 70 °C for one hour. 24 μl of 20 mg/ml proteinase K solution was added to the resulting reaction solution, and the resulting solution was incubated at 50 °C for one hour. To the resulting reaction solution was added an equal volume of phenol, followed by agitation. The resulting solution was left to stand overnight at 4 °C, to extract DNA into the aqueous layer. The aqueous layer was then recovered. To the aqueous layer was added an equal volume of phenol/chloroform, followed by agitation and extraction for 2 hours, and the resulting aqueous layer was recovered. To the resulting aqueous layer was added an equal volume of chloroform/isoamyl alcohol, followed by agitation and extraction for 30 minutes, and the resulting aqueous layer was recovered. To the aqueous layer

was added a 2-fold volume of ethanol, to precipitate DNA. The resulting precipitate was dissolved in 300 µl of TE buffer, and the resulting solution was used as chromosomal DNA.

Example 2

Probe-labeling

According to the method described in Current Protocols in Molecular Biology, chromosomal DNA was prepared from Escherichia coli strain W3110. The DNA primer of SEQ ID NO: 17 and the DNA primer of SEQ ID NO: 18, corresponding to parts of the gene coding for the β subunit of Escherichia coli F_0F_1-ATPase, were synthesized using the DNA synthesizer of Model 8905, manufactured by Perceptive Biosystems, Co. Using the synthesized DNAs as primers and the chromosomal DNA of Escherichia coli strain W3110 as a template, the probe was labeled by PCR DIG Probe Synthesis Kit (manufactured by Roche Diagnostics KK). Labeling reaction was carried out according to the manual of the kit, using 0.1 µg of the chromosomal DNA and 0.5 pmol of each of the primers in 50 µl of the reaction solution, and reaction step of 94 °C for 30 seconds, of 55 °C for one minute and of 72 °C for one minute were repeated 30 times.

Example 3

Southern hybridization

10 μg of the chromosomal DNA as isolated in Example 1 was completely digested with restriction endonuclease EcoRI. Similarly, the chromosome was thoroughly cleaved with BamHI. 1 μg each of the samples cleaved with the respective restriction endonucleases was subjected to agarose gel electrophoresis. After electrophoresis, the DNA was transferred onto nylon membrane according to the method described in Molecular Cloning, Second edition.

Hybridization was carried out by using DIG Luminescent Detection Kit (manufactured by Roche Diagnostics KK.). The nylon membrane (Hybond N+) with the DNA transferred thereon was subjected to prehybridization in 1ml of prehybridization solution [0.5 mol/liter Na_2HPO_4-$12H_2O$ (pH7.2), 7 % SDS, 1 mmol/liter EDTA] per 10 cm^2 of membrane at 65 °C for 30 minutes. Then, 1 ml of a prehybridization solution containing 1 μl of the probe prepared in Example 2 per 3 ml of the solution was used per 5 cm^2 of membrane, for hybridization at 65 °C for 16 hours. After the hybridization, the membrane was washed with a wash buffer [40 mmol/liter Na_2HPO_4-12 H_2O (pH 7.2), 1 % SDS] at 65 °C for 20 minutes. The wash procedure was repeated three times. Then, the treatment using 1 ml of DIG Buffer 1 [100 mmol/liter Tris-HCl (pH7.5), 150 mmol/liter NaCl] per 2 cm^2 of membrane at room temperature for 10 minutes was repeated twice. Subsequently, blocking against antibodies was carried out, using 0.5 w/v % blocking solution at room

temperature for one hour. Labeling with an antibody was carried out at room temperature, using 62.5 µl of DIG Buffer 1 containing 75 mU/ml anti-DIG AP Fab fragment and 0.2 % Tween 20 per 1 cm^2 of membrane for 30 minutes. Additionally, wash procedure using DIG Buffer 1 containing 0.2 % Tween 20 at a ratio of 0.125 ml per 1 cm^2 of membrane at room temperature for 15 minutes was repeated twice. Subsequently, the membrane was treated with DIG Buffer 3 [100 mmol/liter Tris-HCl (pH9.5), 100 mmol/liter NaCl, 50 mmol/liter $MgCl_2$] for 3 minutes. After dropwise addition of CSPD solution, the resulting mixture was incubated at 37 °C for 15 minutes, luminescent reaction was promoted.

After termination of the luminescent reaction, the membrane was dried in air. Subsequently, the membrane was exposed to an X-ray film for 30 minutes.

As a result of hybridization, the probe strongly hybridized with the EcoRI-cleaved 6.5-Kb fragment and the BamHI-cleaved 6-Kb fragment of Corynebacterium ammoniagenes chromosomal DNA.

Example 4

Colony hybridization

1 µg of Corynebacterium ammoniagenes ATCC6872 chromosomal DNA was completely digested with restriction endonuclease EcoRI or BamHI, and the individual digested

fragments were separated by agarose gel electrophoresis to recover fragments around the EcoRI-cleaved 6.5-kb fragment and the BamHI-cleaved 6-Kb fragment, by RECOCHIP (manufactured by Takara Shuzo Co., Ltd.). 0.1 μg of a plasmid vector pBluescript II KS (-) (manufactured by Stratagene) was thoroughly digested with EcoRI or BamHI, which was then subjected to dephosphorylation reaction with temperature-sensitive alkaliphosphatase (manufactured by GIBCO BRL).

The EcoRI cleavage fragment around 6.5 kb and the EcoRI-cleaved and phosphatase-treated pBluescript II KS (-) were subjected to a ligation reaction at 16 °C for 16 hours, using a ligation kit. Using the ligation reaction solution, Escherichia coli strain JM109 was transformed by the method using calcium ion described in Molecular Cloning, Second edition. The resulting transformant was spread on an LB agar medium containing 100 μg/ml ampicillin, for overnight culturing at 37 °C. Similarly, the BamHI cleavage fragment around 6 kb and the BamHI-cleaved and phosphatase-treated pBluescript II KS (-) were subjected to a ligation reaction at 16 °C for 16 hours, using a ligation kit. Using the ligation reaction solution, the Escherichia coli strain JM109 was transformed by the method described above. The resulting transformant was spread on an LB agar culture medium containing 100 μg/ml ampicillin, for overnight culturing at 37 °C. The

growing colony was transferred on the membrane (Hybond N+) and lysed to fix the DNA on the membrane according to the method described in Molecular Cloning, Second edition. Colony hybridization was carried out by the same method as for Southern hybridization in Example 3.

Consequently, a bacterial strain harboring the plasmid pE61 carrying the 6.5-kb EcoRI cleavage fragment of Corynebacterium ammoniagenes ATCC6872 chromosomal DNA and a bacterial strain harboring the plasmid pDW31 carrying the 6-kb BamHI cleavage fragment thereof were selected as positive clones.

From the colonies of the two clones were isolated the plasmids, which were the plasmids pE61 and pDW31, so that the structures of the plasmids were confirmed by digestion with restriction endonucleases (Fig. 1).

Example 5

Recovery of upstream gene

The plasmids pE61 and pDW31 as obtained in Example 4 were found not to carry the genes predicted to be present upstream among the genes coding for the proteins composing the F_0F_1-ATPase protein complex. Therefore, the genes present upstream were isolated by the following method.

The DNA primer having the nucleotide sequence represented by SEQ ID NO: 19 and the DNA primer having the

nucleotide sequence represented by SEQ ID NO: 20, corresponding to parts of the F_0F_1-ATPase b subunit gene which exists in the plasmid pE61, were synthesized using a DNA synthesizer of Model 8905, which was manufactured by Perceptive Biosystems, Co. Using the synthesized DNAs as primers and the plasmid pE61 DNA as a template, the probe was labeled by PCR DIG Probe Synthesis Kit (manufactured by Roche Diagnostics). Using the resulting probe, Southern hybridization was carried out by the same method as in Example 3. Strong hybridization with a 5.0-Kb HindIII-digested fragment of the chromosomal DNA of Corynebacterium ammoniagenes strain ATCC6872 was observed.

Using a bacterial strain harboring a plasmid constructed by inserting a fragment of the HindIII-digested chromosome DNA around 5.0 kb into pBluescript II KS (-), colony hybridization was carried out. Then, a bacterial strain harboring a plasmid pUH71 carrying a Hind III fragment of 5-kb was hybridized strongly and thus, was selected as a positive clone.

From the colony of the clone was isolated the plasmid, which was named pUH71. Then, the structure of the plasmid was confirmed by digestion with restriction endonucleases.(Fig. 1)

Example 6

Determination of a nucleotide sequence

The nucleotide sequences of the inserted fragments in the plasmids pE61 and the pDW31 and in the plasmid pUH71 were determined with ABI 377 Sequencer. Open reading frames consisting of individual nucleotide sequences represented by each of SEQ ID NOS: 9 to 16 encoding the amino acid sequences represented by each of SEQ ID NOS: 1 to 8, respectively, existed in the nucleotide sequences of the fragments.

As a result of comparative analysis the nucleotide sequences of the fragments with other bacterial F_0F_1-ATPase genes, it is shown that the nucleotide sequences correspond to an operon of genes of the subunits a, c, b, δ, α, γ, β and ε located in this order, as in many other bacteria. The nucleotide sequence of the operon is shown as SEQ ID NO: 21.

Further, Table 1 shows the amino acid sequence identity (%) of each subunit of Bacillus subtilis F_0F_1-ATPase [J. Bacteriol., 176, 6802 (1994)] and each subunit of Escherichia coli F_0F_1-ATPase[Biochem. J., 224, 799 (1984)] with each subunit of the F_0F_1-ATPase of the present invention, respectively.

Table 1

	subunits							
	a	b	c	α	β	γ	δ	ε
Bucillus subtilis	26	29	47	54	65	35	24	31
Escherichia coli	23	31	35	48	61	38	24	32